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# Covalent Binding of Poly(Ethylene Glycol) (PEG) to the Surface of Red Blood Cells Inhibits Aggregation and Reduces Low Shear Blood Viscosity

**Jonathan K. Armstrong, Herbert J. Meiselman, and Timothy C. Fisher***

Department of Physiology and Biophysics, University of Southern California School of Medicine, Los Angeles

**A simple method to coat human red blood cells (RBC) with PEG is described. Using a reactive derivative, monomethoxy-PEG (mPEG) was covalently attached to the surface of RBC in aqueous media under mild conditions. The PEG coating dramatically reduced aggregation and low shear viscosity of RBC resuspended in autologous plasma, and inhibited RBC agglutination by blood group-specific antibodies. Morphology and deformability of the PEG-treated cells were unaltered. The PEG coating of the RBC surface may be of significant benefit in the treatment of a variety of diseases characterized by vaso-occlusion or impaired blood flow, e.g., myocardial infarction, sickle cell disease. Am. J. Hematol. 56:26–28, 1997.** 

**Key words: poly(ethylene glycol); bioconjugates; erythrocyte; aggregation; viscosity**

## INTRODUCTION

Poloxamer 188 (P188), a PEG-containing block copolymer, is an effective inhibitor of RBC aggregation and reduces blood viscosity in vitro [1]. A pharmaceutical preparation of P188 (RheothRx® injection) has been shown to improve blood flow in ischemic tissues [2] and to reduce myocardial infarct size in animal models [3]. Recent clinical studies have demonstrated significant potential for RheothRx® in the treatment of myocardial infarction [4] and sickle cell crisis [5].

The poloxamer molecule consists of two hydrophilic poly(ethylene glycol) (PEG) chains connected by a hydrophobic poly(propylene glycol) (PPG) core. The mechanism of action of P188 appears to result from adsorption of the PPG core onto the RBC surface, with the hydrophilic PEG ''arms'' extending outward from the cell surface, forming a steric barrier which inhibits RBC aggregation and consequently reduces low shear blood viscosity [1]. The adsorption of the PPG core is weak and non-specific, thus a relatively high plasma concentration of P188 (>1 mg/ml) is needed to achieve a significant reduction of RBC aggregation. As P188 undergoes rapid renal clearance from the circulation ($t_{1/2} = 5$ hr), a continuous intravenous infusion of 30–60 mg/kg/hr is required to maintain a therapeutic plasma level [4], which amounts to a total dose of 50 g/day or more.

We reasoned that similar inhibition of RBC aggregation and reduction of blood viscosity would be achieved more efficiently if PEG could be directly attached to the RBC surface. Thus we have developed a simple method to covalently bind PEG to RBC. This treatment is more effective than P188 as an inhibitor of RBC aggregation in vitro, and has the theoretical advantages that only milligram quantities of PEG are required to adequately cover the surfaces of the whole circulating RBC population, and that a single treatment should be sustained for the lifetime of the RBC.

## MATERIALS AND METHODS

Monomethoxy-poly(ethylene glycol), molecular mass 5,000 g/mol, activated with cyanuric chloride (mPEG-$C_3N_3Cl_2$), was purchased from Sigma (St. Louis, MO). P188 (Pluronic F68) was a generous gift from BASF Corporation, Parsippany, NJ. After informed con-

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*Correspondence to: Dr. Timothy C. Fisher, Department of Physiology and Biophysics, University of Southern California School of Medicine, 1333 San Pablo Street, Los Angeles, CA 90033. e-mail: fisher@hsc.usc.edu

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sent, blood was drawn from 5 healthy adult volunteers and anticoagulated with EDTA. The RBC were washed with Dulbecco's PBS (pH 7.4, Sigma) and then resuspended at 50% hematocrit in 30 mM phosphate buffered saline containing 10% autologous plasma (pH 8.0). An aliquot of a fresh solution of mPEG-$C_3N_3Cl_2$ was added to the RBC suspension (final polymer concentration 5 mg/ml). An equivalent volume of PBS was added to control samples. After gentle mixing at 25°C for 1 hr, RBC were washed twice with PBS and reconstituted with autologous plasma to 40% hematocrit. The viscosity of the reconstituted PEG-treated blood was measured over a range of shear rates using a Contraves LS30 low-shear viscometer (Contraves AG, Zürich, Switzerland). RBC aggregation was studied with a Myrenne Aggregometer (Myrenne GmGH, Roetgen, Germany) [6]. RBC morphology was examined by optical microscopy, and RBC deformability was assessed using the Cell Transit Analyzer [7]. RBC agglutination was examined semi-quantitatively by incubation of PEG-treated and control RBC with serial dilutions of anti-A, anti-B, and anti-D specific blood grouping reagents (Dade, Baxter Diagnostics Inc., FL) in a 96-well plate.

## RESULTS

The viscosity of RBC in plasma at 40% hematocrit as a function of shear rate is shown in Figure 1. The curve for untreated, control RBC demonstrates the well-established shear-dependent decrease in viscosity: At low shear the viscosity is markedly elevated due to RBC aggregation, while with each stepwise increase in shear, the viscosity decreases due to the disruption of RBC aggregates [8]. RBC treated with mPEG showed a much reduced low shear viscosity (75% less than control). By comparison, P188 at 5 mg/ml was less effective, reducing the low shear viscosity by approximately 30%. RBC aggregation measured by the Myrenne aggregometer (M mode) was reduced by 93 ± 8% after mPEG-treatment compared to 33 ± 9% for 5 mg/ml P188 (mean ± sd). Microscopic examination of RBC in autologous plasma showed that >98% of RBC remained as biconcave discocytes after mPEG-$C_3N_3Cl_2$ treatment. The only observable microscopic difference between mPEG-coated and control RBC was the absence of rouleaux formation. No change in RBC deformability was detected with the Cell Transit Analyzer.

RBC agglutination by anti-D ($Rh_0$) was completely prevented by mPEG treatment (three Rh+ donors), while an up to 8-fold increase was observed in the titre required to induce visible agglutination by anti-A and anti-B antibody.

## DISCUSSION

The use of reactive PEG intermediates has recently been widely applied to modify synthetic surfaces, proteins, liposomes, and drugs [9,10]. The PEG coating of these substances (''PEGylation'') has enabled prolonged circulatory times [9], increased biocompatibility [10], and reduced immunogenicity [9]. However, most PEGylation techniques require highly non-physiological conditions and, thus, there are few previous studies investigating the direct bonding of PEG to living cells.

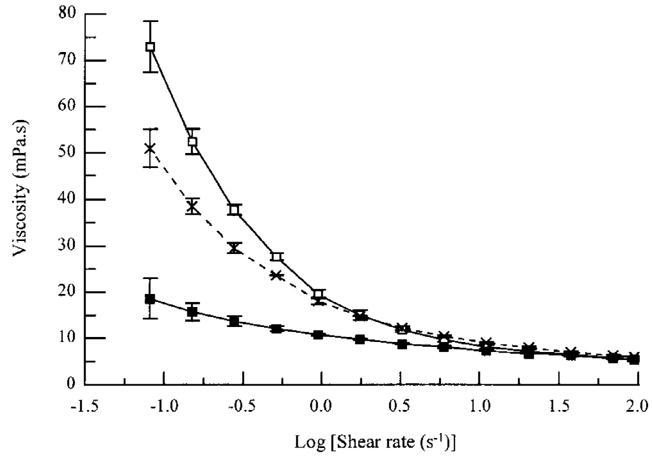


**Fig. 1. Viscosity of reconstituted blood as a function of shear rate at 40% hematocrit and at 25°C. Control RBC (—□—), RBC with poloxamer 188 (- - -X- - -) added to the plasma at 5 mg/ml, mPEG-coated RBC (—■—). Data are mean ± sd for 5 donors.**

The method presented here provides a simple means to coat RBC surfaces with PEG under mild, near-physiological conditions with no apparent adverse effects on RBC morphology or deformability. The treatment reduced RBC aggregation and blood viscosity more effectively than P188, the most potent inhibitor of RBC aggregation currently in clinical use, and may thus have potential applications for the treatment of ischemic vascular disorders, notably myocardial infarction and vaso-occlusive crisis in sickle cell disease. A propitious application would be for resuscitation after hemorrhagic shock. P188 has been shown to reduce mortality after acute hemorrhage and retransfusion in rabbits [11]. Given that transfusion is required, the use of PEG-treated RBC would not only restore oxygen-carrying capacity, but the reduction of low shear viscosity should help to improve blood flow in underperfused tissues.

PEGylation of the RBC surface also inhibited agglutination by antibodies against blood group antigens; partially for A and B and completely for Rh (anti-D). This result has been independently confirmed in other recent studies [12,13] in which several other blood group antigens were also found to be masked. Inhibition of antibody binding by PEG-coating the RBC prior to transfusion could theoretically prolong the survival of transfused erythrocytes in patients with existing alloantibodies, or reduce the incidence of alloimmunization in multiply-transfused patients. While increased RBC survival alone would be

beneficial for various kinds of chronic anemia, the combination of reduced blood viscosity and enhanced RBC survival could be especially advantageous for the treatment of patients with sickle cell disease, in whom ischemia rather than anemia is the primary cause of morbidity.

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